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14. ABSTRACT
We hypothesized that dehydroepiandrosterone metabolites or their synthetic derivatives are able to bind to the androgen receptor with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We previously identified three potential compounds with marginal androgenic activity. We also demonstrated that, using multiple prostate cancer lines, these compounds could inhibit androgen-induced growth of androgen receptor-positive cells *in vitro* but that treatment with each compound resulted in modest decreases in tumor growth in mouse models for prostate cancer. We here assessed whether the dehydroepiandrosterone derivatives altered androgen-mediated androgen receptor functions, including androgen receptor mRNA/protein stability, androgen receptor N/C-terminal interaction, androgen receptor/androgen receptor coregulator interactions, and androgen receptor nuclear translocation, in prostate cancer cells. All three compounds were found to interrupt interactions between N-terminus and C-terminus of androgen receptor as well as androgen receptor and several androgen receptor coregulators but failed to affect the stability and nuclear translocation of androgen receptor.

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Table of Contents

Introduction

Although antiandrogens that can block androgen action through the androgen receptor (AR) have been widely used for the treatment of prostate cancer, the majority of available ones possess agonist activity, resulting in increases in serum prostate-specific antigen (PSA) levels, known as the antiandrogen withdrawal syndrome [1, 2]. In addition, we previously found that androstenediol, a physiological metabolite from dehydroepiandrosterone (DHEA) and a precursor of testosterone, has an intrinsic androgenic activity which was not completely antagonized by two antiandrogens clinically used, flutamide and bicalutamide (BC) [3]. Therefore, new and more effective antiandrogenic compounds with marginal androgenic activities need to be identified. Our hypothesis in the current project was that DHEA metabolites or their synthetic derivatives are able to bind to the AR with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We previously screened DHEA derivatives/metabolites for their androgenic and antiandrogenic activities and found that three compounds, 3β-acetoxyandrost-1,5-diene-17-ethylene-ketal (ADEK), 3β-hydroxyandrost-5,16-diene (HAD), and 3-oxo-androst-1,4-diene-17-ketal (OADK), show only marginal agonist effects and suppress significantly 5α-dihydrotestosterone (DHT)- and androstenediol-induced AR transcriptional activities [4-6]. Thus, ADEK, HAD, and OADK have the potential to function as potent antiandrogens that carry fewer risks of withdrawal response if used for therapy in patients with prostate cancer.

We have subsequently assessed the effects of ADEK, HAD, and OADK on prostate cancer progression *in vitro* and *in vivo* and have demonstrated the data indicating that these DHEA derivatives inhibit androgen-induced growth of prostate cancer cells. The tasks in the approved Statement of Work in this period [months 49-53; owing to grant transfer to the current institution, no funding was available during the first 7 months (July 1, 2013 – February 2, 2014)] would be to determine the mechanisms of how the compounds inhibit prostate cancer growth (*Task 3* for months 37-60; Tasks *3-c*, *3-d*, *3-e*, *3-f*, and *3-h*).

Body

Effects of ADEK, HAD, and OADK on AR stability

We previously reported that ADEK, HAD, and OADK inhibited androgen-induced expression of AR in LNCaP and CWR22Rv1 cells while they did not reduce AR expression in the absence of androgens [7 & annual report, July 2011]. To further determine whether the DHEA derivatives affect the stability of AR mRNA and protein, quantitative reverse transcription (RT)-polymerase chain reaction (PCR) and Western blotting analyses were performed in LNCaP and CWR22Rv1 cell lines pretreated with actinomycin D or cycloheximide. In these experiments, however, there were no significant differences in the ratios of AR expression/degradation between the control versus ADEK/HAD/OADK groups in the presence and absence of DHT. These findings suggest that the DHEA derivatives have little influence on AR stability in prostate cancer cells.

Effects of ADEK, HAD, and OADK on AR NH_2-/COOH-terminal (N/C) interaction

It is well documented that AR N/C interaction is important for full AR activation [8]. We therefore assessed whether the DHEA derivatives exert an influence on the interaction, using mammalian two-hybrid assay, in AR-negative prostate cancer cells. PC-3 and DU145 cells were transfected with a GAL4-hybrid plasmid expressing AR-DNA binding domain/ligand binding domain, a VP16-hybrid plasmid expressing AR-NH_2-terminus, and a luciferase reporter plasmid (pG5-Luc), and treated with DHT and each antiandrogenic compound (Fig. 1). As expected, DHT induced AR N/C interactions in both cell lines. DHEA derivatives only marginally increased the luciferase activity (except HAD in PC-3; 4.5-fold over mock treatment, $P < 0.05$) and significantly reduced DHT-enhanced activities. Thus, it was likely, as seen in BC, that ADEK, HAD, and OADK inhibited androgen-mediated AR N/C interactions in prostate cancer cells.

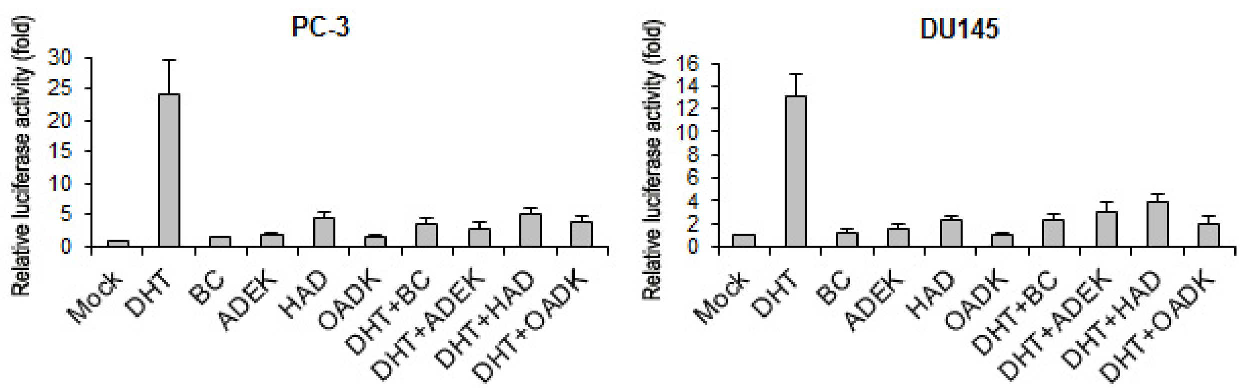

Figure 1. The impact of DHEA derivatives on AR N/C interaction. PC-3 or DU145 cells were transfected with pCMX-GAL4-AR-C, VP16-AR-N, pG5-Luc, and pRL-TK, and subsequently cultured in the presence or absence of 1 nM DHT, 10 µM BC, 1 µM ADEK, 1 µM HAD, and/or 1 µM OADK, as indicated. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit and luminometer. Luciferase activity is presented relative to that of mock treatment in each cell line. Each value represents the mean + standard deviation from four independent experiments.

Effects of ADEK, HAD, and OADK on AR-AR coregulator interactions

It has also been known that maximal or proper androgen action via AR requires the interactions between AR and selective AR coregulators [9]. We therefore assessed whether the DHEA derivatives exert an influence on the interactions, using mammalian two-hybrid assay, in prostate cancer cells. DU145 cells were transfected with a GAL4-hybrid plasmid expressing AR-DNA binding domain/ligand binding domain, a VP16-hybrid plasmid expressing each AR coregulator, and a luciferase reporter plasmid (pG5-Luc), and treated with DHT and each antiandrogenic compound (Fig. 2). As expected, DHT induced AR interaction with each AR coregulator. As reported [9-11], BC also promoted the interactions between AR and ARA70 or ARA54 (but not between AR and ARA55 or SRC-1 in our assays). Similarly, DHEA derivatives significantly ($P < 0.05$) induced some of the interactions (ADEK: ARA70 and ARA54; HAD: ARA70, ARA54, and ARA55; and OADK: ARA70 and ARA54). Nonetheless, these compounds inhibited all of the DHT-induced interactions. Thus, it was likely, as seen in BC, that ADEK, HAD, and OADK inhibited androgen-mediated interactions between AR and AR coregulators in prostate cancer cells, while they also had agonist effects.

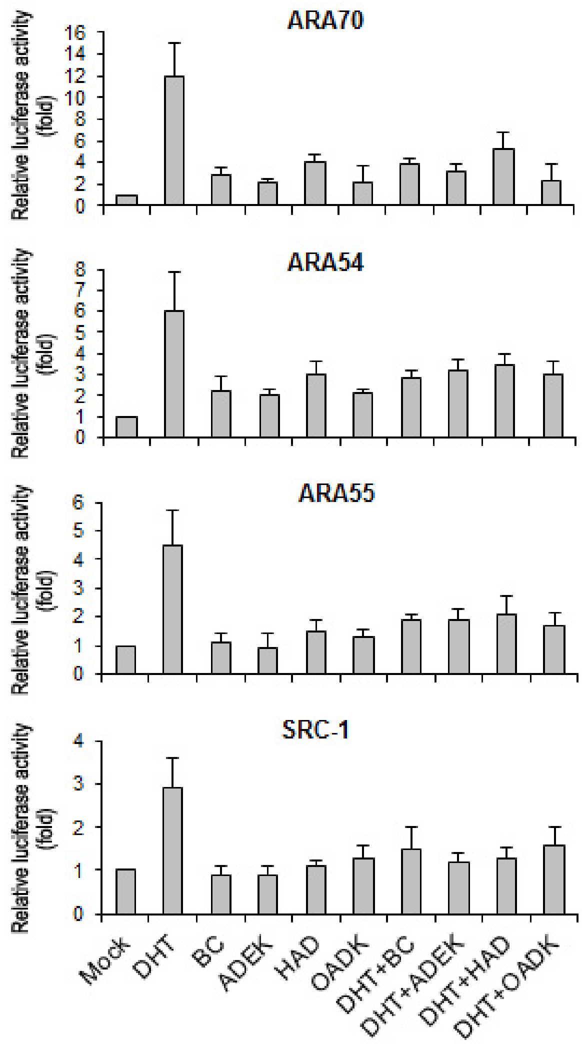

Figure 2. The impact of DHEA derivatives on AR-AR coregulator interactions. DU145 cells were transfected with pCMX-GAL4-AR-C, VP16-ARA70/ARA54/ARA55/SRC-1, pG5-Luc, and pRL-TK, and subsequently cultured in the presence or absence of 1 nM DHT, 10 µM BC, 1 µM ADEK, 1 µM HAD, and/or 1 µM OADK, as indicated. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit and luminometer. Luciferase activity is presented relative to that of mock treatment. Each value represents the mean + standard deviation from at least three independent experiments.

Effects of ADEK, HAD, and OADK on nuclear translocation of AR

Immunofluorescent staining was performed to assess the effects of DHEA derivatives on nuclear translocation of AR in prostate cancer cells. PC-3 cells cultured in the presence or absence of DHT, ADEK, HAD, and/or OADK were subjected to immunofluorescence with an anti-AR antibody. As described in BC [12], DHEA derivatives did not strongly block the receptor nuclear translocation induced by DHT. These findings are being confirmed by subcellular fractionation of nuclear and cytoplasmic proteins followed by Western blotting.

Key Research Accomplishments

1. (for *Tasks 3-c* & *3-d*) ADEK, HAD, and OADK had little influence on the stability of AR mRNA/protein in prostate cancer cells.

2. (for *Task 3-e*) ADEK, HAD, and OADK were found to inhibit AR N/C interaction induced by androgen in prostate cancer cells.

3. (for *Task 3-f*) ADEK, HAD, and OADK were found to inhibit interactions between AR and selective AR coregulators induced by androgen in prostate cancer cells.

4. (for *Task 3-h*) ADEK, HAD, and OADK failed to block nuclear translocation of AR induced by androgen in prostate cancer cells.

Reportable Outcomes

Peer-reviewed publications derived from the current award

1. Kawahara T, **Miyamoto H*** (corresponding author): Androgen receptor antagonists in the treatment of prostate cancer. *Clin Immunol Endocr Metab Drugs* (August 1, 2013; Epub ahead of print).

Additional peer-reviewed publications

2. Kakiuchi Y, Choy B, Gordetsky J, Izumi K, Wu G, Rashid H, Joseph JV, **Miyamoto H*** (corresponding author): Role of frozen section analysis of surgical margins during robot-assisted laparoscopic radical prostatectomy: a 2,608-case experience. *Hum Pathol* 44(8): 1556-1562, 2013 (August).

3. Ishiguro H, Kawahara T, Li Y, **Miyamoto H*** (corresponding author): "Anti-tumor activities of dexamethasone" in Dexamethasone: Therapeutic Uses, Mechanism of Action and Potential Side Effects (Sauvage A/Levy M Eds.), pp.117-135, Nova Science Publishers, Hauppauge, New York, 2013 (August) (ISBN 978-1-62808-406-1).

4. Fang L-Y, Izumi K, Lai K-P, Liang L, Li L, **Miyamoto H**, Lin W-J, Chang C: Infiltrating macrophages promote prostate tumorigenesis via modulating androgen receptor-mediated CCL4-STAT3 signaling. *Cancer Res* 73(18): 5633-5646, 2013 (September).

5. Venigalla S, Wu G, **Miyamoto H*** (corresponding author): The impact of routine frozen section analysis during partial nephrectomy on surgical margin status and tumor recurrence: A clinicopathologic study of 433 cases. *Clin Genitourin Cancer* 11(4): 527-536, 2013 (December).

6. <u>Izumi K, Li Y, Ishiguro H, Zheng Y, Yao JL, Netto GJ, **Miyamoto H*** (corresponding author): Expression of UDP-glucuronosyltransferase 1A in bladder cancer: association with prognosis and regulation by estrogen. *Mol Carcinogen* 54(4): 314-324, 2014 (April)</u> (This article acknowledges the current award).

7. Li Y, Ishiguro H, Kawahara T, Kashiwagi E, Izumi K, **Miyamoto H*** (corresponding author): Loss of GATA3 in bladder cancer promotes cell migration and invasion. *Cancer Biol Ther* 15(4): 428-435, 2014 (April).

8. Slavin S, Yeh C-R, Da J, Yu S, **Miyamoto H**, Messing EM, Guancial EA, Yeh S: Estrogen receptor α in cancer-associated fibroblasts suppresses prostate cancer

invasion *via* modulation of thrombospondin 2 and matrix metalloproteinase 3. *Carcinogenesis* 35: 1301-1309, 2014 (June).

9. Lin S-J, Lee SO, Lee Y-F, **Miyamoto H**, Yang D-R, Li G, Chang C: TR4 nuclear receptor functions as a tumor suppressor of prostate tumorigenesis *via* modulation of DNA damage/repair system. *Carcinogenesis* 35: 1399-1406, 2014 (June).

10. Izumi K, Lin W-J, **Miyamoto H**, Huang C-K, Maolake A, Kitagawa Y, Kadono Y, Konaka H, Mizokami A, Namiki M: Outcomes and predictive factors of prostate cancer patients with extremely high prostate-specific antigen level. *J Cancer Res Clin Oncol* 140: 1413-1419, 2014.

11. Ishiguro H, Kawahara T, Zheng Y, Netto GJ, **Miyamoto H*** (corresponding author): Reduced glucocorticoid receptor expression predicts bladder tumor recurrence and progression. *Am J Clin Pathol* 142: 157-164, 2014.

12. Ishiguro H, Kawahara T, Zheng Y, Kashiwagi E, Li Y, **Miyamoto H*** (corresponding author): Differential regulation of bladder cancer growth by various glucocorticoids: corticosterone and prednisone inhibit cell invasion without promoting cell proliferation or reducing cisplatin cytotoxicity. *Cancer Chemother Pharmacol* (June 1, 2014; Epub ahead of print).

Invited Speakers

1. Chang Gung University College of Medicine (Department of Urology), Kaohsiung, Taiwan (June 2014)

2. Chang Gung University Memorial Hospital at Kaohsiung Medical Center (Department of Pathology), Kaohsiung, Taiwan (June 2014)

3. Educational Lecture for at the 55th Annual Spring Meeting of the Japanese Society of Clinical Cytology, Yokohama, Japan (June 2014)

Conclusion

We have attempted to clarify molecular mechanisms of how DHEA derivatives suppress androgen-mediated growth of prostate cancer cells by investigating their impact on AR functions (*Task 3*). During this period, we performed real-time RT-PCR, Western blotting, mammalian two-hybrid assay, and immunofluorescence. Our data in prostate cancer cells demonstrate that ADEK, HAD, and OADK: 1) have little influence on the stability of AR mRNA/protein; 2) inhibit AR N/C interaction induced by androgen; 3) inhibit interactions between AR and selective AR coregulators, including ARA70, ARA54, ARA55, and SRC-1, induced by androgen; and 4) fail to block nuclear translocation of AR induced by androgen. We will further explore the mechanisms responsible for the suppression of prostate cancer growth by ADEK, HAD, and OADK, as well as by additional DHEA derivatives that were described in the original Project Narrative; Specific Aim 1, Alternative approach 1 and showed significant inhibitory effects on prostate cancer cell proliferation and PSA expression *in vitro* [7 & annual report, July 2010].

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